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Identification of early inflammatory genes involved in adenosine signaling using a pharmacogenomics approach

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List of abbreviations

ADA	Adenosine deaminase
AMP	Adenosine monohosphate
ATP	Adenosine triphosphate
AR	Adenosine receptor
BAL	Bronchoalveolar lavage
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
CGS-21680	9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine
CNS	Central nervous system
CREM	cAMP response element modulator
Ct	Threshold cycle
KD	Kilo Dalton
DAPI	4',6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
cDNA	Complementary DNA
ELISA	Enzyme-Linked ImmunoSorbent Assay
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptors
HMC-1	Human mast cell line 1
IB-Meca	1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-y l]-N methyl-b-D-ribofuranuronamide
IgE	Immunoglobulin E
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
LPS	Bacterial lipopolysaccharide
LUC	Luciferase, firefly luciferase
MAPKs	Mitogen-activated protein kinase/s
MC	Mast cell
MCP-1	Monocyte chemoattractant protein 1
MIP-1β	Macrophage Inflammatory Protein-1 beta
NBRE	Nerve-growth factor responsive element
NECA	5′-N-Ethylcarboxamido-adenosine
NK	Natural killer cells
NR4A	Nuclear orphan receptor 4 A
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PKA	Protein kinase A
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
RNA	Ribonucleic acid
mRNA	Messenger RNA
Rpm	Revolution per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SCH-58261	7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine)

SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TBS	Tris-buffered saline
TBST	Tris-buffered saline Tween® 20
TLR4	Toll-like receptor 4
UT	Untreated

Summary

Adenosine is a paradoxical inflammatory modulator. It can both participate in inflammatory shutdown and also mediate the persistence of inflammation, by activating different adenosine receptors (AR). Here, a pharmacogenomics approach was employed to identify AR sub-type specific cellular signals in the human mast cell-line 1 (HMC-1). Two gene products previously not associated to adenosine signaling were further characterized. First, we determined that AR activation results in upreguation and secretion of macrophage inflammatory protein-1 beta (MIP-1β) into the cell culture medium, suggesting a mechanism by which adenosine can modulate chemotaxis by mast cells. Second, we detected a sharp upregulation of the inflammatory transcription factors NR4A2 and NR4A3, accompanied by an increase in their transcriptional activity. Notably, this effect appears to be mediated by A_{2B}AR and A_3AR, while A_{2A}AR activation counteracted NR4A2 and NR4A3 induction by other ARs. Furthermore, while non-selective AR activation strongly potentiated PMA/I-mediated induction of NR4As, selective A_{2A}AR engagement partially revoked it, indicating that A_{2A}AR modulatory effect is conserved to other pro-inflammatory stimuli. Overall, these findings show that AR activation can have broad regulatory effects on human mast cells, not only by a direct effect on inflammation-associated factors, but also by influencing the recruitment of inflammatory cells through chemotactic cytokines.

Introduction

General concepts

Adenosine is an endogenous by-product normally present at low concentrations in the extracellular space. An increment in adenosine formation occurs as a result of hypoxia, tissue injury and acute or chronic inflammation. The rapid accumulation of adenosine is followed by a number of responses, which can be regarded as organ protective. On one hand, extracellular adenosine represents a pre-eminent alarm molecule that reports tissue injury in an autocrine and paracrine manner to surrounding tissue. On the other hand, it decreases tissue energy demand via a direct inhibitory effect on parenchymal cell function and by providing a more favorable cellular environment by increasing nutrient availability via vasodilatation (Linden, 2001). In addition, adenosine helps to maintain tissue integrity by modulating the immune system and by regulating exuberant immune responses. During chronic inflammatory processes, however, the sustained formation of adenosine has been associated with deleterious effects. Elevated adenosine concentrations can be found, for example, in bronchoalveolar lavage (BAL) and exhaled breath condensate of human patients with asthma (Driver et al., 1993;Huszar et al., 2002), where it perpetuates inflammation and contributes to airway hyperresponsiveness.

Although the bioavailability of adenosine is an important determinant of its biological functions, the pattern of expression and distribution of its receptors in the anatomical–structural sites accounts for the observation that adenosine may exert either deleterious or protective roles. Each of the four adenosine receptors (AR) identified to date, A_1, A_{2A}, A_{2B} and A_3, has a unique pharmacological profile, tissue distribution and effector coupling. The characterization of each receptor subtype has stimulated the search for specific ligands able to modulate the effects of this molecule in a directed way. To date, several compounds of different chemical classes with great potential have been identified but side effects in several organs derived from the broad distribution of the receptors across the organism have prevented their clinical application.

Adenosine homeostasis

Adenosine is composed of a molecule of adenine attached to a ribose sugar molecule (ribofuranose) moiety via a glycosidic bond. It derives as a by-product of adenosine triphosphate (ATP) degradation during the cycle of energy generation, principally by the cleavage of adenosine monophosphate (AMP) by 5'-nucleotidases, both in the intracellular and extracellular compartments. Once in the extracellular space, adenosine binds to G-protein coupled adenosine receptors, activating alternate signaling cascades, which ultimately result in biological effects. In physiological conditions, the level of adenosine is kept low by its conversion to AMP by adenosine kinase (Arch and Newsholme, 1978) and it is also degraded to inosine and hypoxanthine by adenosine deaminase (Trams and Lauter, 1974).

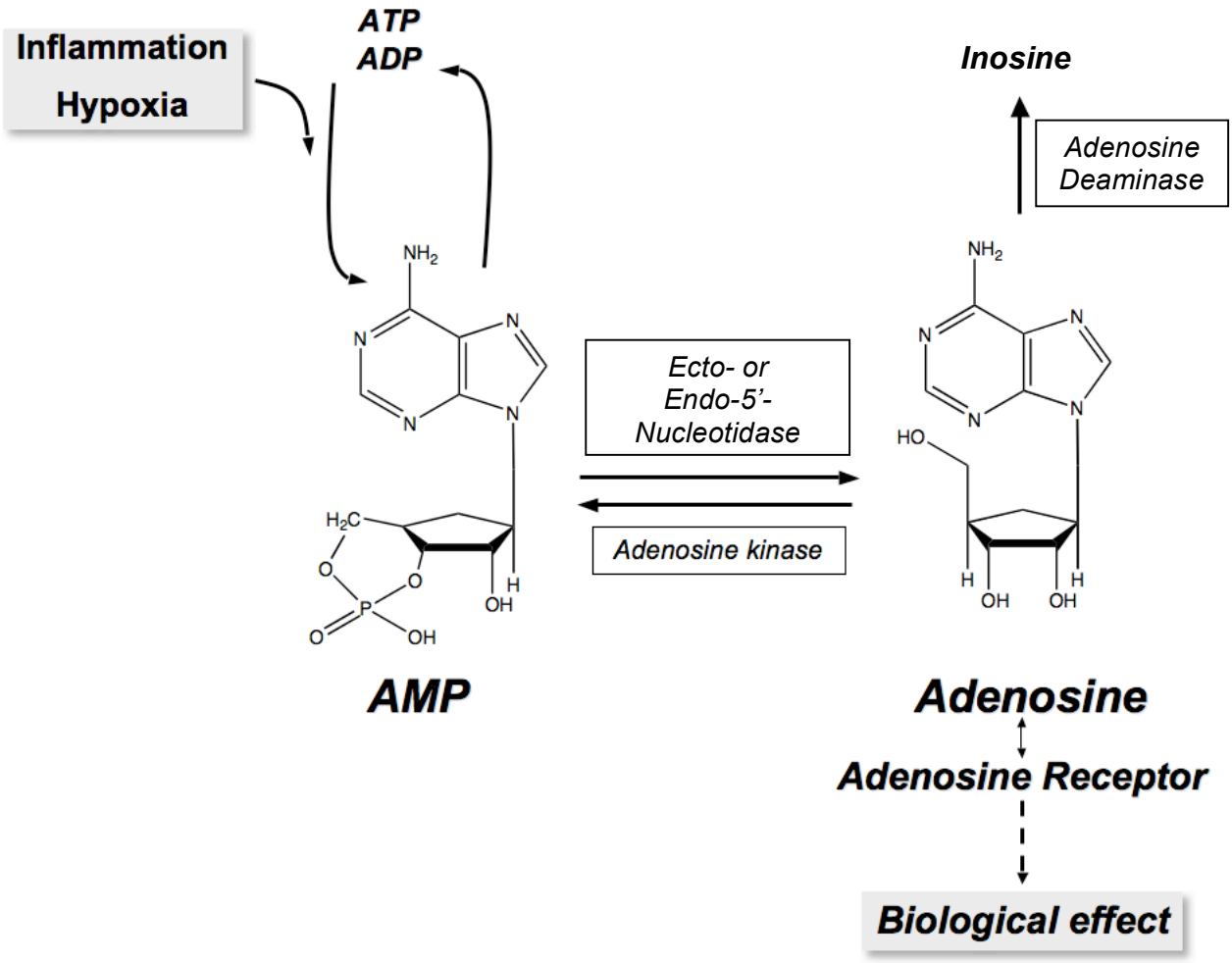

Fig 1. Adenosine metabolism.

In metabolically stressful conditions such as ischemia, sepsis and hypoxia, extracellular adenosine concentration increases in proportion to energy consumption, ATP degradation and AMP accumulation, reaching local concentrations of up to 30 μM – a 150-fold increase over basal levels (Van Belle et al., 1987).

Biological functions of adenosine

Different functions have been attributed to adenosine in different organs. In the central nervous system adenosine acts as a neuromodulator, integrating a wide variety of brain functions. It modulates CNS excitability and plays a role in mechanisms of seizure susceptibility (Pagonopoulou et al., 2006), sleep induction (Basheer et al., 2004), basal ganglia function (Schwarzschild et al., 2006), pain perception (Sawynok and Liu, 2003), cerebral blood flow (Shi et al., 2008) and respiration (Lahiri et al., 2007). Consequently, dysfunction of the adenosine system is involved in pathologies ranging from epilepsy to neurodegenerative disorders and psychiatric conditions. Because this nucleoside can integrate and fine-tune glutamatergic and dopaminergic neurotransmission, adenosine-regulating agents have the potential to modify a wide range of downstream effects. In addition, adenosine receptors are inhibited by caffeine and other methylxanthines and provide a potential target for treatment of cerebral ischemia (Ribeiro, 2005), seizures (During and Spencer, 1992), pain, Parkinson's disease (Schapira et al., 2006) and Huntington's disease (Blum et al., 2003).

Adenosine is a potent vasodilator in most vascular beds (with the exception of renal afferent arterioles and hepatic veins) and it is indicated for myocardial perfusion scintigraphy in humans. This nucleoside also shows marked angiogenic effects, which are mostly mediated by the induction of pro-angiogenic factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). In addition, this nucleoside shows anthiarrythmic effects (reduction of both heart rate and contractility) and it is intravenously administered to restore normal heart rhythm in patients with paroxysmal supraventricular tachychardia. Adenosine also protects

the myocardium (as well as other tissues) from hypoxia or ischaemia through preconditioning (recently reviewed by Das and Das, 2008).

Extracellular adenosine in the kidney affects vascular and tubular functions. It shows a prominent glomerular filtration rate reducing effect, by activating A_1AR on afferent arterioles (Hansen and Schnermann, 2003). In the deep cortex and medulla, adenosine-induced vasodilation contributes to minimize medullary injury during hypoxic episodes. AR activation can also inhibit the secretion of renin (Edlund et al., 1994). Adenosine-mediated prevention of ischemia-reperfusion injury in the kidney is a clinically relevant protective mechanism and the underlying molecular events are now starting to be elucidated.

A role for adenosine in glucose and insulin metabolism has also been established. Activation of the A_1AR has been associated with an increase in plasma insulin, increase in insulin sensitivity and glucose uptake (Dong et al., 2001). Agonists for this AR subtype have been tested in human as adjuvant therapy in type 2 insulin resistant diabetes and resulted in improved tryacilglycerol levels and reduced fatty acid levels (Donnelly and Qu, 1998).

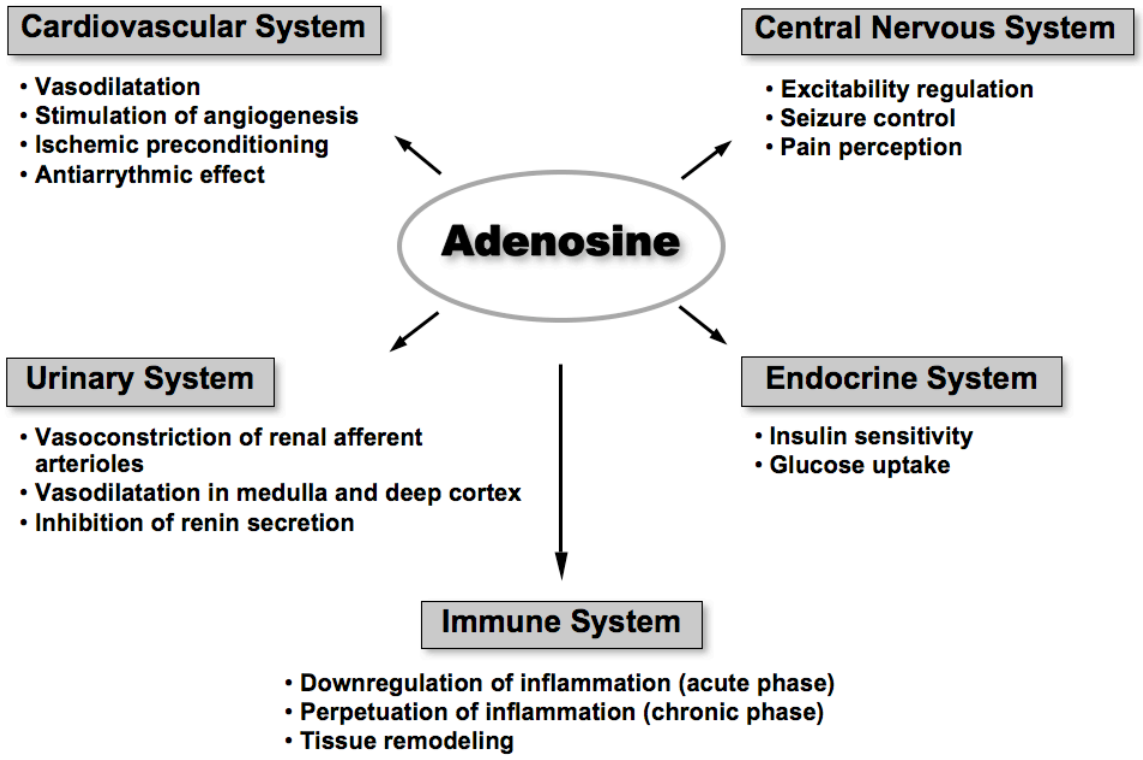

Fig 2. Main AR-mediated effects on several systems

Adenosine has well described anti-inflammatory and tissue protective effects in situations such as ischemia-reperfusion injury and acute inflammation (Day et al., 2004). In chronic conditions such as asthma and chronic obstructive pulmonary disease adenosine can, however, play a pro-inflammatory and tissue-destructive role and contribute to remodeling of the airways (Blackburn, 2003). These seemingly paradoxical effects can be explained by the activation of alternate ARs on inflammatory cells, which activate diverse signaling cascades and different effector pathways.

Adenosine receptors and their role in inflammation

The adenosine G-coupled receptors (GPCR) consist of a central core domain with seven transmembrane helices connected by three intracellular and three extracellular loops. Both transmembrane and extracellular regions of ARs form the ligand-binding pocket and several amino acids that contribute to the ligand-binding properties have been identified via mutagenesis (Moro et al., 2006).

The four distinct adenosine receptors, A_1, A_{2A}, A_{2B} and A_3 have been cloned in rodents, dog, sheep, cow and humans. Usually, more than one adenosine receptor is expressed in a single cell resulting in a composite pharmacological response. ARs are densely expressed on virtually all inflammatory cell types, including monocytes and macrophages (Hasko et al., 2007; Khoa et al., 2006), dendritic cells (Panther et al., 2001), mast cells (Feoktistov and Biaggioni, 1995), neutrophils (Fortin et al., 2006), platelets (Gessi et al., 2000), endothelial cells (Li et al., 1998), eosinophils (Kohno et al., 1996), epithelial cells (Sitaraman et al., 2001) and fibroblasts (Chan et al., 2006), as well as lymphocytes (Apasov et al., 2000), NK and NKT cells (Harish et al., 2003;Lappas et al., 2006).

A_1AR

This subclass of AR has been associated with both pro- and anti-inflammatory effects. On one hand, it has been shown that activation of A_1 receptor can promote activation of human neutrophils and monocytes, thus leading to

proinflammatory responses (Merrill et al., 1997; Salmon et al., 1993). On the other, the genetic removal of the A_1 receptor gene in adenosine deaminase-deficient mice resulted in enhanced pulmonary inflammation, indicating that in this context the activation of A_1AR serves an anti-inflammatory and/or protective role in the regulation of pulmonary disorders (Sun et al., 2005). A_1 receptors were also shown to play an anti-inflammatory and protective role in experimental models of injury in the heart, nerves and kidney (Lee et al., 2004;Liao et al., 2003;Tsutsui et al., 2004). More recently, a role for A_1AR in angiogenesis induction has been suggested (Clark et al., 2007). A_1AR activation inhibits the adenylyl cyclase (G_i-mediated). Other pathways involved include protein kinase C (PKC), phosphoinositide 3 (PI3) kinase and mitogen-activated protein (MAP) kinases (reviewed by Jacobson and Gao, 2006).

A_{2A}AR

A_{2A}AR receptors are widely distributed in the body. High expression of adenosine A_{2A} mRNA has been found in leukocytes and platelets and in some areas of the central nervous system, while intermediate levels have been found in lung and heart (Fredholm et al., 2001). A_{2A}AR are co-expressed with A_{2B}AR in human mast cells found in bronchoalveolar lavage (Feoktistov and Biaggioni, 1998; Suzuki et al., 1998). Several lines of evidence suggest that in the lung A_{2A}AR activate a protective mechanism playing a critical role in the down-regulation of inflammation and tissue damage in different models (Ohta and Sitkovsky, 2001; Thiel et al., 2005). Activation of A_{2A} receptors seems to affect multiple aspects of the inflammatory process, modulating neutrophil activation and degranulation, oxidative species production, adhesion molecules expression, cytokine release and mast cell degranulation (Lappas et al., 2005). Therefore, it is not surprising that selective adenosine A_{2A}AR agonists may hold potential in the pharmacological control of airway inflammation and inflammation-mediated lung tissue injury.

A_{2A}AR signal mainly by activation of the adenylate cyclase (cAMP), protein kinase A (PKA) canonical pathway, but they can also signal through the activation of an exchange factor that is directly activated by cAMP (Epac) (Fredholm et al., 2007).

A_{2B}AR

Functional human A_{2B}ARs have been identified in the smooth muscle cells (Zhong et al., 2004), lung fibroblasts (Zhong et al., 2005), endothelial cells (Feoktistov et al., 2002), bronchial epithelium (Clancy et al., 1999), and mast cells (Feoktistov and Biaggioni, 1998). A_{2B}AR is a low-affinity receptor, requiring higher concentrations of adenosine to be significantly activated. Engagement of this receptor subtype has been associated to pro-inflammatory biological events, including vasodilation (Martin, 1992), facilitation of antigen-induced degranulation in human mast-cells (Feoktistov and Biaggioni, 1995) and increased release of interleukin-6 and monocyte chemoattractant protein 1 (MCP-1) from epithelial cells, astrocytes and fibroblasts (Sitaraman et al., 2001; Zhong et al., 2005). Also, A_{2B}AR signalling in the human mast cell line HMC-1 augments interleukin-8 (IL-8) release, upregulates Th2 cytokines (IL-4 and IL-13) and promotes IgE synthesis by B lymphocytes (Ryzhov et al., 2004). Also, ADA-/- mice treated with the A_{2B} antagonist CVT-6883 showed less pulmonary inflammation, fibrosis, and alveolar airspace enlargement than controls (Sun et al., 2006). The A_{2B}AR couples both to G_s, promoting cAMP accumulation, and to G_q triggering Ca^{2+} mobilization and activation of PLC and MAPKs (Gao et al., 1999); the arachidonic acid pathway has also been implicated in A_{2B} signaling (Donoso et al., 2005). Interestinlgy, crosstalk between G_s and G_q appears to regulate IL-4 production by A_{2B} in human mast cells (Ryzhov et al., 2006).

A_3AR

In humans, a relatively high density of functionally active A_3ARs can be found in eosinophils and elevated transcript levels of this AR have been detected in lung biopsies of patients with asthma and in activated lymphocytes (Gessi et al., 2004). The functional role of this AR subtype remains controversial, mainly due to interspecies differences. In rats, mast cell degranulation seems to be dependent on A_3AR activation (Fozard et al., 1996; Shepherd et al., 1996) and A_3 receptor antagonism attenuates pulmonary inflammation, reduces eosinophil infiltration and decreases airway mucus production (Young et al., 2004). In contrast, activation of these receptors by the A_3AR specific agonist IB-MECA in humans inhibits eosinophil chemotaxis and other proinflammatory

functions (Walker et al., 1997). In addition, this compound induces anti-inflammatory effects in experimental animal models of collagen- and adjuvant-induced arthritis. In this context a combined therapy of A_3AR agonist and methotrexate in adjuvant-induced arthritis in rats yielded an additive anti-inflammatory effect (Ochaion et al., 2006). New and more potent A_3AR agonists are currently being developed, some of which are already in clinical trials for the treatment of rheumatoid arthritis (Silverman et al., 2008). The signaling pathway associated with A_3AR activation comprises G_i-mediated inhibition of adenyly cyclase and G_q-mediated stimulation of PLC (Gessi et al., 2004).

Adenosine signaling in lung inflammatory conditions

Several findings suggest that adenosine plays a critical role in the pathogenesis of chronic inflammatory disorders of the airways such as asthma and chronic obstructive pulmonary disease. First, adenosine administered by inhalation was shown to be a powerful bronchoconstrictor of asthmatic but, importantly, not of normal airways (Cushley et al., 1983). Second, increased formation of adenosine occurs in chronically inflamed airways as demonstrated by elevated levels of adenosine both in BAL fluid and exhaled breath condensate of asthmatic patients with elevations correlating with the degree of inflammatory insult (Driver et al., 1993; Huszar et al., 2002). Third, blockade of adenosine re-uptake by dipyridamole increased the bronchoconstrictor response to adenosine in asthma indicating that accumulation of extracellular adenosine was closely associated with the asthmatic airway response (Cushley et al., 1985). Finally, a recent study has established that the administration of adenosine 5'-monophosphate provoked airflow limitation in dogs with cadmium chloride-induced airway inflammation but not on healthy individuals (Hirt et al., 2007).

This has led to the current hypothesis that release of histamine and other spasmogens from resident airway mast cells may account for the bronchoconstrictor response to inhaled adenosine in asthma. Mast cells have been traditionally associated with allergic reactions through IgE engagement

on the high affinity FceRI receptors, leading to degranulation of cytoplasmic granules and release of biologically potent mediators (i.e. histamine). In addition, adenosine (as well as bacterial and viral antigens, cytokines and neuropeptides among others) can provoke the secretion of inflammatory mediators from these cells, in a selective process that is independent of degranulation. Recently, mast cells have been reported to mediate adenosine-induced airway hyperresponsiveness in a degranulation-independent fashion (Hua et al., 2008). Both adenosine mediated mast cell degranulation and differential secretion of inflammatory mediators (degranulation-independent) have raised great interest and are being widely explored.

Aim of this work

Numerous experiments conducted on experimental animal models sustain the potential of selective AR activation in the treatment of inflammation and inflammation-related diseases, but this potential has failed to translate into novel therapies. For this reason, the further development of AR-based strategies of immunomodulation requires knowing the exact molecular mechanisms that mediate inhibition/stimulation of inflammatory processes by adenosine. The aim of this work is to identify and characterize cellular signals triggered by activation of specific ARs implicated in pro and anti-inflammatory signaling employing a pharmacogenomics approach.

Material and Methods

Cell culture

The human mast cell line-1 (HMC-1) was a kind gift from Dr. J. H. Butterfield, Mayo Clinic, Rochester, MN, USA. These cells were cultured at 37 °C in saturated humid atmosphere under 5 % CO2 in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) supplemented with 10 % fetal bovine serum with iron (Invitrogen), 1.2 mM Alphathiglycerol (Sigma) and 100'000 units/l Penicillin and 100mg/l Streptomycin (complete IMDM). Subculturing was done when the cells reached a concentration of 10^6 cells/ml (every third day). They were split in a ratio of 1:2.

Cell stocks were obtained by centrifuging the cultured cells (10 minutes, 1200 rpm) and resuspending them with freezing media (complete media as described above with 5% DMSO) to reach a density of around 5 Mio cells/ml. Aliquots were slowly frozen until they reached -80°C and then transferred to liquid nitrogen. To start a new culture, the aliquots were thawed to 37°C, transferred into falcon tubes (Greiner), diluted with pre-warmed complete medium and collected by centrifugation (10 minutes, 1200 rpm). Cells were then resuspended with pre-warmed complete medium and transferred into T75 flasks (75cm2, Nunc A/S).

Cell treatments

All chemicals were obtained from Sigma-Aldrich (Switzerland) unless otherwise indicated. HMC-1 cells were seeded at 8 x 10^5 cells/ml and allowed to settle overnight. They were then treated with concentrations from 0.1µM to 100µM of the following chemicals: the adenosine analogue NECA (5′-N-Ethylcarboxamido-adenosine), the selective adenosine receptor A2A agonist CGS-21680 (9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine) and the selective adenosine receptor A2A antagonist SCH-58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) or a combination of NECA and SCH-58261. The length of treatment varied between 30 minutes and 72 hours (specific treatment schemes are indicated together with the results). Cells were pretreated with 1

U/ml adenosine deaminase (Roche) to remove any pre-existing endogenous adenosine for 20 minutes.

Cell viability assay

HMC-1 cells were seeded in a 96-wp and allowed to recover overnight before exposing them to increasing amounts of NECA, CGS-21680 and SCH-58261 for 24 hours. The metabolic activity was quantified after different treatment times using the redox indicator dye Alamar Blue (Invitrogen). After incubation, the fluorescent signal was measured by the Luminescence Spectrometer LS55 (PerkinElmer) with an excitation wavelength of 560nm and an emission wavelength of 590nm. The recorded values were corrected for unspecific fluorescence (background) and the decrease in cell viability was calculated in reference to the untreated control (100%).

Genome wide gene expression analysis

Global gene expression analysis was done in 4 independent experiments, consisting of the treatment with 10 µM NECA and 10 µM CGS-21680, and untreated cells as a baseline control.

After 3h treatment of HMC-1 cells total RNA was extracted as described in the following section. The quality of RNA was determined on the Agilent Lab-on-a-chip Bioanalyzer 2000 (Palo Alto, CA, USA). Samples with a total area under 28S and 18S bands of less than 65% of total RNA, as well as a 28S/18S ratio of less than 1.5, were considered to be degraded and therefore excluded from microarray analysis. cDNA was then synthesized as described in the following section, and complementary RNA (cRNA) labeled with the IVT labeling kit (Affymetrix). cRNA quality was assessed with the Agilent Lab-on-a-chip Bioanalyzer 2000. The biotin-labeled cRNA was fragmented and hybridized on Human Genome U122 plus 2.0 microarrays (Affymetrix) following the manufacturer's instructions. After hybridization periods of 16 hours the microarrays were automatically washed and stained on the Affymetrix Fluidics Station 450. Staining of the hybridized probes was performed with fluorescent streptavidin-phycoerythrin conjugates (1 mg/ml; Molecular Probes). The subsequent scanning of DNA microarrays was carried

out on an Affymetrix scanner 3000 7G. The generated data was then normalized and analyzed using the GeneSpring 7.3.1 sofware (Agilent, Palo Alto, CA, USA). We employed pair-wise analysis based on B - Fabric infrastructure tool (Functional Genomics Center, ETH/University of Zurich, Switzerland) and the GeneGo Metacore (www.genego.com) integrated software for data mining and functional analysis of experimental data. The gene ontology database (www.geneontology.org) was also consulted for the molecular function of each transcript.

Real time polymerase chain reaction (RT-PCR)

Cells were collected by centrifugation at the corresponding time points and total RNA was recovered using the Qiashredder and Rneasy mini kit (Qiagen). Concentration and quality of total RNA were measured with the Ultraspec 2100 pro spectrophotometer (Amersham Biosciences). Samples with a UV absorbance 260/280 ratio of 1.8 to 2.1 were considered to be suitable for cDNA synthesis. The RNA samples were stored at -20°C until use. Complementary DNA (cDNA) was synthesized using the one-cycle cDNA synthesis kit followed by a sample cleanup to optimize volumes and concentration of the cDNA (GeneChip sample cleanup module, Affymetrix). Specific primers for the selected transcripts as well as TaqMan probes were obtained from Applied Biosystems. 40 ng of cDNA were mixed with 90nM of forward and reverse primers and supplemented with 25nM of the corresponding TaqMan probe in a final volume of 20 µl. The reactions were performed in a 7500 Fast Real-time PCR-System ABI 7500 (Applied Biosystems) in 40 cycles (95 °C for 3 sec, 60 °C for 30 sec) after an initial 20 sec incubation at 95 °C, and was analyzed with the 7500 Fast System SDS Software System (Applied Biosystems). The fold change in expression of each gene was calculated with the 2-Delta Delta C(T) method (Livak and Schmittgen, 2001). Briefly, this method compares Ct values of the samples to Ct values of the untreated controls. For a given well, the threshold cycle (Ct) represents the PCR cycle at which the software first detects a noticeable increase in reporter fluorescence above a baseline signal. Each of these

values had been normalized to an appropriate endogenous housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blot

Preparation of whole cell lysates

HMC-1 at a concentration of 10^6 cells/ml were harvested after treatment to the indicated concentrations of the corresponding chemicals for varying incubation times, collected by centrifugation, washed with 1x PBS and lysed with 180µl 1x lysis buffer (50mM Tris, 150mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate and a protease inhibitor cocktail tablet (Roche, Switzerland) per 25 to 50 ml buffer). After lysis, the samples were sonicated (5 cycles, 10 seconds, 70% power; Banderlin Sonopuls) and centrifuged to remove the insoluble fraction.

Supernatants were aliquoted and stored at -20°C until use.

Protein quantification

Protein concentration was determined by the Bradford method. Briefly, 2ul of lysate was incubated with 1 ml of 1x Bio-RAD Protein Assay (Bio-Rad Laboratories) for 5 minutes in the dark. Absorbance at 595 nm was then determined on an Ultraspec Plus spectrophotometer (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Three BSA standards (0.2, 0.5, 0.9 ug/ml) were employed to construct a calibration curve against which the protein concentration of the samples was calculated by extrapolation.

SDS-Page and Western Blot analysis

30µg of protein in 1x Laemmli buffer were separated on a 10% polyacrylamide gel by standard SDS-PAGE technique (Laemmli, 1970), using a Bio-Rad vertical electrophoresis unit. Electrophoresis was accomplished at 35 mA per gel for 1 hour in 25mM Tris, 125mM Glycin, pH 8.6, containing 0.1% SDS. A protein size marker ladder to show different known sizes of proteins was used with every SDS gel (Precision Plus Protein All Blue Standards, Biorad). After electrophoresis, proteins were transferred onto Immune-Blot polyvinylidene difluoride (PVDF) membranes (0.2 µgm pore

size, Bio-Rad) using the Bio-Rad tank transfer system. The PVDF membranes were soaked in methanol and equilibrated in transfer buffer (25mM TRIS, 125 mM Glycine, pH 8.6, containing 0.025% SDS). The transfer was carried out at 60V for 1 hour at 4°C.

After transfer, the membranes were incubated for 16 hours at 4°C with blocking solution (5% non-fat dry milk, 3% BSA in TBST (10mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4) to block any unspecific binding sites. NR4A2 antibody (Santa Cruz Biotechnology) was employed as primary antibody at a dilution of 1:2000 with 2.5% BSA in TBST. Incubation was carried out in glass tanks for 1 hour at room temperature on an orbital shaker. In a similar fashion, GAPDH (Ambion, USA) antibody diluted 1:10'000 with 2.5% BSA in TBST was employed for 30 min as an internal normalization control. Following the incubation with the primary antibody the membranes were washed 3 times for 10 minutes in 3% non-fat dry milk in TBST and then incubated for 1 hour with the appropriate peroxidase conjugated secondary antibodies (anti-rabbit IgG for NRA2; anti-mouse IgG for GAPDH, both diluted 1:80'000) in 3% non-fat dry milk in TBST. Then, the membranes were washed in 3% non-fat dry milk in TBST followed by TBST and TBS (3x 10 min). Finally, enhanced chemiluminescence was performed with SuperSignal West Femto Maximum (Pierce/ Socochim, 5 minutes) and images were acquired on a LAS-3000 (Fujifilm Life Science, Japan). The intensity of the NR4A2 signals was normalized against the GAPDH content in each sample.

Luciferase reporter gene assay

The 4xNBRE-Luc reporter plasmid was a kind gift from H. Harant. This vector carries the firefly luciferase gene under control of the NBRE (NGFI-B responsive element) sequence (AAGGTCA). 1.5 x 10^6 HMC-1 cells in 1ml were seeded into a 6 well plate and were co-transfected with the 4xNBRE-Luc reporter and the pRL-TK vector (Promega), which constitutively expresses the renilla luciferase gene. The ratio of co-transfection 4xNBRE-Luc: pRL-TK was 30:1 and Lipofectamine 2000 (Invitrogen) was employed as transfction ragent. 4 hours post transfection, the medium was changed and the cells were allowed to recover for 24 hours before exposure to the indicated

concentrations of the selected chemicals. Following exposure, cells were collected by centrifugation at varying times, washed once with 1x PBS and lysed with 250 µl of lysis buffer (Promega). Lysis was completed by agitating for 20 minutes on an orbital shaker followed by centrifugation for 1 minute at 13'200 rpm to remove the insoluble fraction. 30 µl of the supernatant were used to determine the expression of firefly and renilla luciferase using the Dual Luciferase system (Promega), as indicated by the manufacturer. Briefly, firefly luciferase activity was first measured by adding Luciferase Assay Reagent II (Promega). After quantification, this reaction is quenched and the renilla luciferase reaction is initiated by adding Stop&Glo Reagent (Promega), which generates a second luminescent signal. Induction of the reporter gene was then calculated from the ratio of activity of the two enzymes. All luminescent measurements were performed automatically in a 96 well-plate in a Luminescence Spectrometer (MLX, Dynex).

MIP-1-β assay

A human MIP-1-β Elisa kit (RayBiotech) was used to determine the level of MIP-1-β protein in the supernatant of HMC-1 cells. HMC-1 cells were seeded at a concentration of 2 x 10^6 cells in 1ml in a 24 well plate. 16 hours later the cells were treated with the indicated concentrations of NECA and CGS-21680 for varying lengths of time. The MIP-1-β assay was performed following the manufacturer's instructions. The plate was read at 450 nm on Mulitskan RC (Thermo Labsystems).

Results

Expression of ARs in HMC-1 cells

HMC-1 cells exhibit a phenotype that is in several aspects similar to tissue resident mast cells. Therefore, we selected this cell line for the evaluation of genome-wide transcriptional reprogramming upon AR activation this cell line. Previous reports showed that HMC-1 cells show expression of functional A_{2A}, A_{2B} and A_3 ARs (Feoktistov et al., 2003). In order to rule out the possibility that mutagenic events resulting from perpetuation of this cell line had affected AR expression, we decided to confirm the expression of these receptors in the cells in culture in our lab. Figure 3 shows the relative abundance of AR subtypes as determined by RT-PCR. In agreement with previous reports, we observed that A_{2B} and A_3 are the predominant AR subtypes, while A_{2A}AR represent less than 5% of ARs expressed in resting HMC-1. As expected, transcripts for A_1AR couldn't be detected.

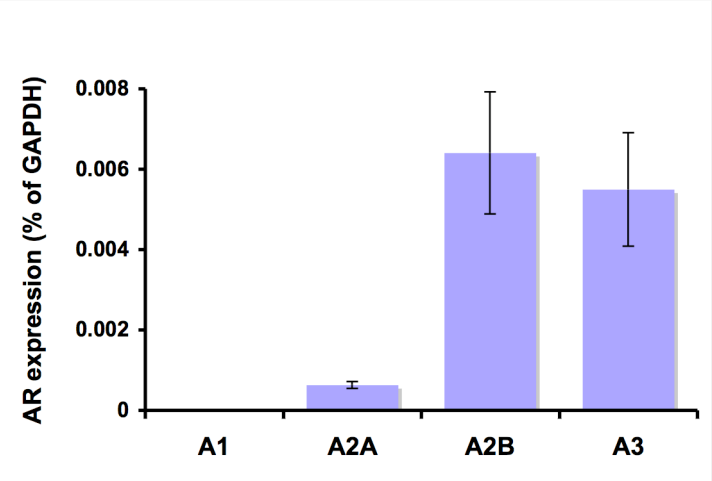

***Figure 3**. RT-PCR analysis of mRNA encoding AR subtypes was performed as described. Values are expressed as percentage GAPDH (house keeping control) and represent the mean of three independent measurements ± SEM.*

Toxicity of AR ligands on HMC-1 cells

Next, we wanted to determine the toxicity threshold of the compounds selected for the pharmacological stimulation of the ARs (NECA, CGS-21680

and SCH-58261). Figure 4 shows the viability of HMC-1 cells with increasing doses of these compounds. While concentrations of NECA of 100μM did not affect cell viability, the same concentration of CGS-21680 reduced it to 75% whereas 100μM of SCH-58261 provoked an even more drastic effect (viability below 10%). On the other hand concentrations of 10μM or lower of each of the three chemicals did not show a toxic effect. The inclusion of 0.5% DMSO in cell culture medium did not affect viability (vehicle control), while 5% of this solvent caused almost a 100% reduction in cell viability.

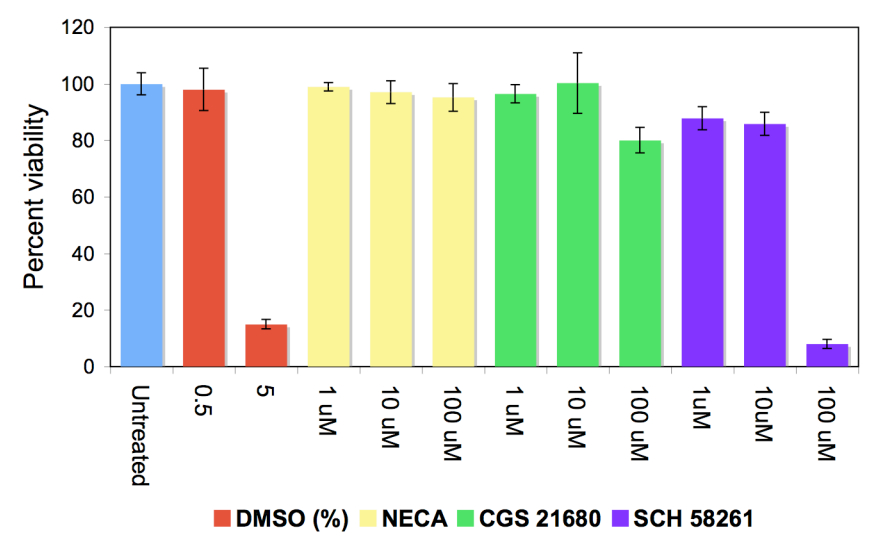

Figure 4. *Viability of HMC-1 cells upon incubation with increasing concentrations of the indicated compounds for 24 hours, expressed as percentage relative to untreated cells.*

Transcriptomics analysis of selective AR activation in HMC-1 cells

In order to identify signals indicative of pro- or anti-inflammatory signaling, selective pharmacological activation of AR subtypes followed by genome-wide profiling was employed. For the analysis of general adenosine signaling, the analogue NECA was selected, while CGS-21680 was preferred for the

identification A_{2A}-specific transcripts. After 3h exposure the cells were collected and processed for the determination of expression profiles that cover sequences of 47'000 human transcripts. The hybridization results were normalized and subsequently filtered using a significance value of $P<0.05$. The overall analysis revealed a strong transcriptional response to NECA, with a set of transcripts common to CGS-21680. Table 1 summarizes the fold changes of the highest upregulated transcripts induced after treatment of HMC-1 cells with NECA or CGS-21680, as compared to untreated controls. The number of transcripts affected by CGS-21680 as well as the intensity of these changes was more moderate than those by NECA.

Gene Name	Description	NECA FC	CGS FC
NR4A2*	Nuclear receptor subfamily 4, group A, member 2	33.52	15.2
STC1*	Stanniocalcin 1	25.37	4.77
NR4A3*	Nuclear receptor subfamily 4, group A, member 3	24.13	13.87
CREM*	CAMP responsive element modulator	13.57	10.04
EGR3	Early growth response 3	10.16	5.44
RGC32	response gene to complement 32	9.54	3.99
HOMER1	Homer homolog 1 (Drosophila)	7.95	4.09
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	6.91	4.05
CGA	Glycoprotein hormones, alpha polypeptide	6.14	2.20
ELL2*	Elongation factor, RNA polymerase II, 2	6.14	3.72
JMY	Junction-mediating and regulatory protein	5.96	2.71
ZEB1	Transcription factor 8 (represses interleukin 2 expression)	5.42	2.75
EGR1	Early growth response 1	5.07	5.63
BCL2L11*	BCL2-like 11 (apoptosis facilitator)	4.99	3.14
ITK	IL2-inducible T-cell kinase	4.76	3.5
PLAUR*	Plasminogen activator, urokinase receptor	4.58	3.4
TP53INP2	p53 induced nuclear protein 2	3.88	2.42
IL2RB	Interleukin 2 receptor, beta	3.78	2.52
EGR2	Early growth response 2 (Krox-20 homolog, Drosophila)	3.72	3.5

Table 1. *Top regulated transcripts by NECA and CGS-21680 treatment ($P<0.05$). NECA FC = Fold changes upon treatment with 10μM of NECA. CGS FC = Fold changes upon treatment with 10μM of CGS-21680. Asterisks indicate transcripts for which two or more set of probes were upregulated.*

An additional group of transcripts became exclusively upregulated upon NECA treatment, including MIP-1β (also known chemokine CCL4; 4.4 fold), CXCR4 (chemokine CXC4 receptor; 3.9 fold), VEGF (vascular endothelial growth factor, 3.35 fold), PLA2G6 (Phospholipase A2, group VI; 3.01 fold) and CASP9 (caspase 9; 3.13x). Among the NECA-specific downregulated transcripts we found IL-7 (-3.6 fold) and UBTF (upstream binding transcription factor RNAS polI; -3.54 fold).

Even though this high dose of CGS-21680 (10μM) is likely to activate not only the A_{2A} but also A_3 receptors on HMC-1, a small group of transcripts appear to be exclusively up- and down-regulated by this treatment, including the NAB2 (NGFI-A binding protein, also known as EGR1 binding protein 1; 2.1 fold), RXRβ (retinoid X receptor beta; -2 fold) and IKBKB (inhibitor of Kappa light polypeptide enhancer in B-cells; 2.05 fold). These transcripts represent interesting potential targets for the modulation of inflammatory/immune responses by adenosine (i.e. the EGR1/NAB2 in the regulation of granulocyte and monocyte lineage commitment and maturation).

Characterization of selected downstream targets

Based on the results of our pharmacogenomics screening, we selected three genes for further study: MIP-1β, which appeared to be exclusively upregulated by NECA and the nuclear orphan receptors NR4A2 and NR4A3.

MIP-1β upregulation

In order to further characterize the induction of MIP-1β we examined the induction of this transcript by real-time PCR. Figure 5 shows the rapid upregulation of MIP-1β after treatment with 10μM NECA, which peaks by 3h and is followed by a decrease to baseline levels within 24h. CGS-21680, in contrast, failed to induce this inflammatory gene product, suggesting that the A_{2A}AR does not contribute to the induction of this protein.

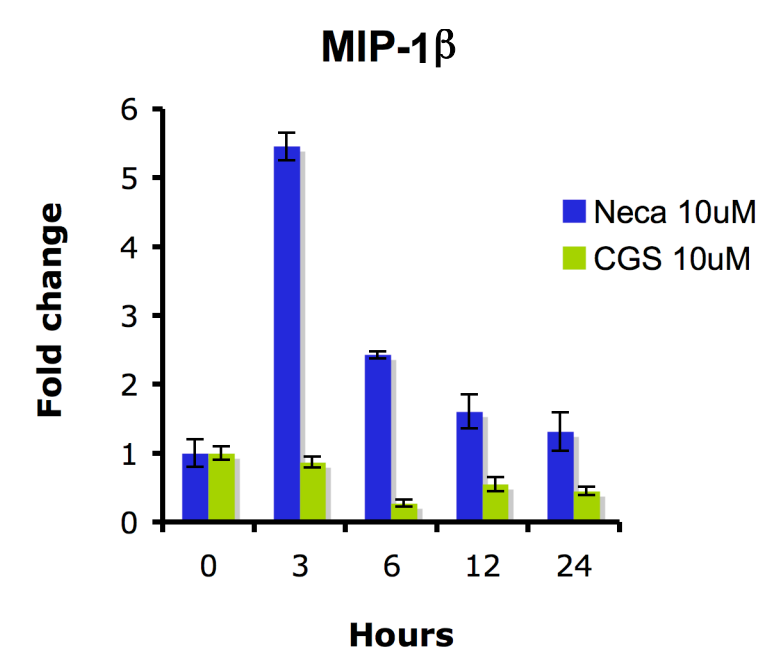

Figure 5. *Time dependent induction of MIP-1β transcript. HMC-1 cells were treated for the indicated times with 10μM of each AR ligand and analyzed by RT-PCR as described. Fold changes are calculated in relation to untreated controls. Values represent the mean of three experiments ± SEM.*

Activation of human cultured mast cells by CD40 ligand was recently shown to stimulate the release of MIP-1β and other cytokines in absence of degranulation (Fischer et al., 2006). Therefore, we wanted to determine whether AR activation could also lead to MIP-1β protein release in HMC-1 cells. For this reason, changes in MIP-1β concentration in cell culture medium were assessed by ELISA 3h after AR activation alone or in combination with LPS. Fig 6 shows that 10μM NECA results in a strong release of MIP-1β from HMC-1 cells, in agreement with the induction of transcription of this gene product (Fig 5). CGS-21680 caused a pronounced but somewhat milder release of MIP-1β. Interestingly, CGS-21680-mediated MIP-1β release does not match the abundance level of its transcript since A_{2A}AR activation does not upregulate MIP-1β transcription (Fig 5). This may imply that transcriptional induction and release of pre-formed MIP-1β are two independent events, and that activation of A_{2A}AR can selectively modulate the release of this chemokine without inducing its transcription. Alternatively, the increment of

MIP-1β in the cell culture medium by CGS-21680 may reflect the activation of ARs other than A_{2A} at this relatively high dose.

Interestingly, the concomitant treatment of HMC-1 cells with NECA and LPS reveals a synergism between these two pathways. While LPS alone did not induce MIP-1β secretion, treatment of HMC-1 cells with LPS and NECA provoked an exaggerated release of this chemokine in the cell culture medium. Additional experiments would be required to determine whether this synergism takes place at the signal transduction level downstream of ARs and toll-like receptor 4 (TLR4, the receptor activated by LPS) or at other levels.

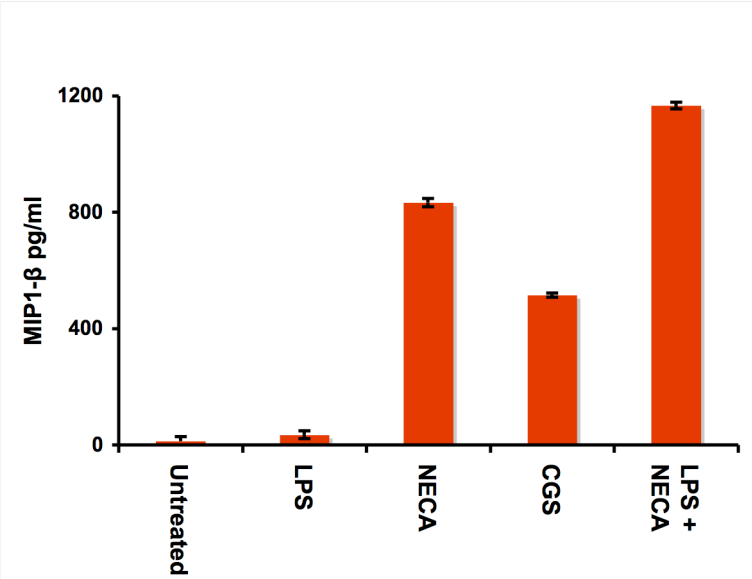

Figure 6. *MIP-1β concentration in cell culture medium. HMC-1 cells were treated for 3h as indicated and cell culture medium analyzed by ELISA. Values represent the mean of three independent measurements ± SEM.*

Nuclear orphan receptors 4A

This subfamily of nuclear orphan receptors is constituted by 3 members: NR4A1 (also known as Nur77 or nerve growth factor inducible B – NGFI-B), NR4A2 (also known as Nurr1) and NR4A3 (also known as NOR1). Two members of this subfamily are among the AR-induced top-regulated transcripts, with their induction being higher after NECA than upon CGS-21680 treatment.

RT-PCR

Figures 7A and 7B show the induction of NR4A2 and NR4A3 within 6 hours of treatment with 1μM CGS-21680, 10μM NECA or the combination 10μM NECA plus 10μM SCH-28261. NECA upregulated NR4A2 and NR4A3 to levels comparable to the detected by the transcriptomics analysis, with maximal induction of 240 fold and 98 fold respectively, 1h after treatment. In contrast, 1μM CGS-21680 (a dose that allows specific A_{2A} activation) failed to induce them. Remarkably, the combined treatment of NECA and SCH-28261, results

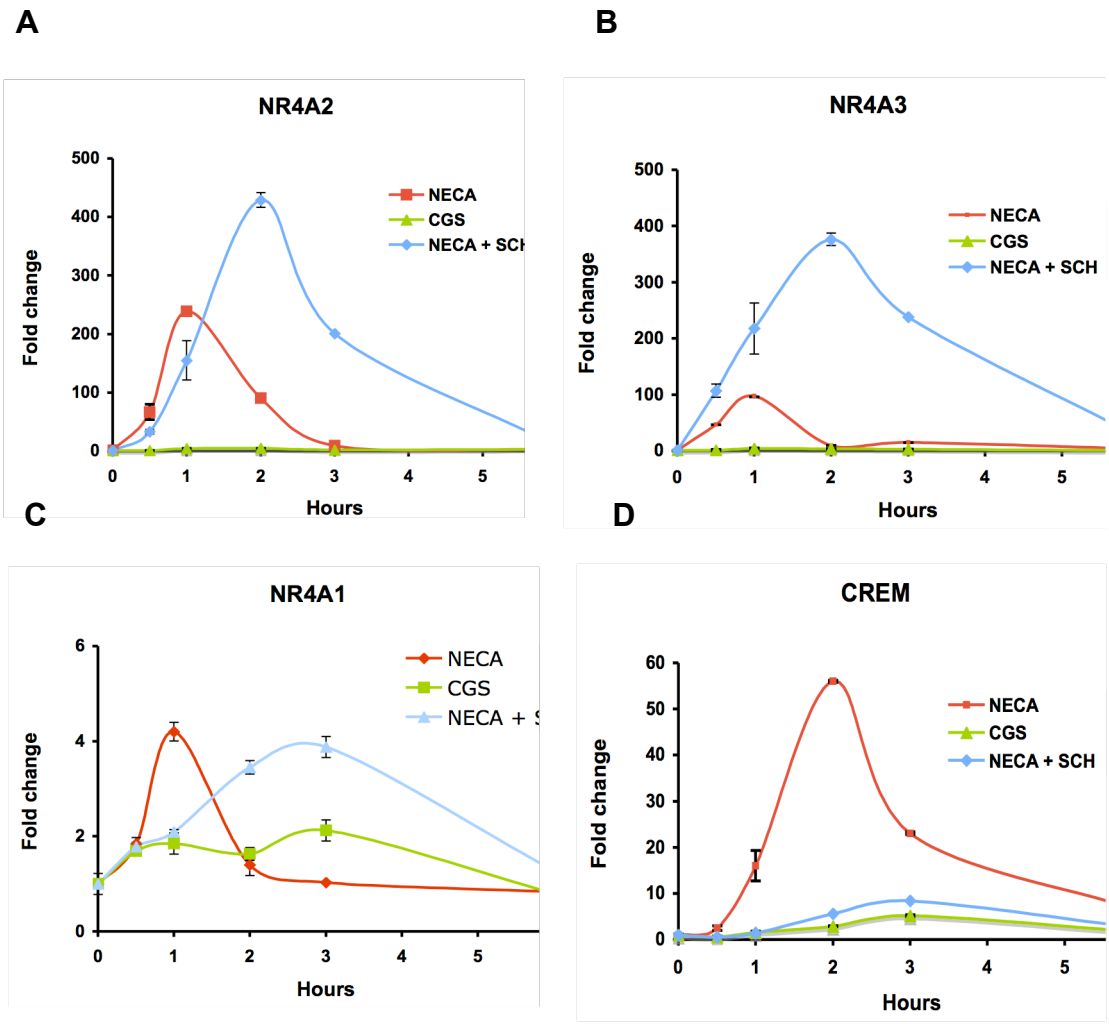

Figure 7. *Time dependent induction of NR4A2 (A), NR4A3 (B), NR4A1 (C) and CREM (D). HMC-1 cells were treated with AR ligands for the indicated times and analyzed by RT-PCR as described. Fold changes are calculated taking untreated samples as baseline. Values represent the mean of three measurements ± SEM.*

in further upregulation of these nuclear orphan receptors, with fold changes up to 429 and 376 over the untreated controls respectively, implying that the induction of these two transcription factors rely on the activation of ARs *other than* A_{2A}.

This interplay between *global* and A_{2A}AR activation is, nevertheless, not conserved to the third member of this family of receptors, the NR4A1: simultaneous treatment with NECA and SCH-28261 does not significantly increase NR4A1's marginal upregulation by NECA (4.2-fold, Fig 7C). In a similar fashion, induction of the cAMP-response element modulator (CREM, a top-regulated gene from the transcriptomics screening) reaches 55-fold change when treated with NECA (Fig 7 D). Treatment with CGS-21680 or the combination of NECA and SCH-58261 induces CREM only marginally, indicating that simultaneous activation of all ARs is required to achieve maximal CREM induction.

NR4A2 protein levels

In the light of these findings, we wanted to determine whether the observed upregulation of NR4A2 and 3 transcripts correlate with changes in protein abundance. Figure 8A shows a western blot analysis of NR4A2 upon treatment with either CGS-21680 or the combination of NECA and SCH-58261. NR4A2 levels increase after NECA and NECA plus SCH-58261 as early as 12h after treatment, returning to normal levels at around 72 h (not shown). However, treatment with CGS-21680 (10μM)□ results in a much weaker upregulation of NR4A2 protein.

To further characterize these changes at earlier time-points, cells were exposed either to NECA or CGS-21680 (1μM) for 3 and 6h (Fig 8B). Interestingly, NECA upregulated NR4A2 protein as early as 3h while selective A_{2A} activation by CGS-21680 caused only a marginal increase in NR4A2.

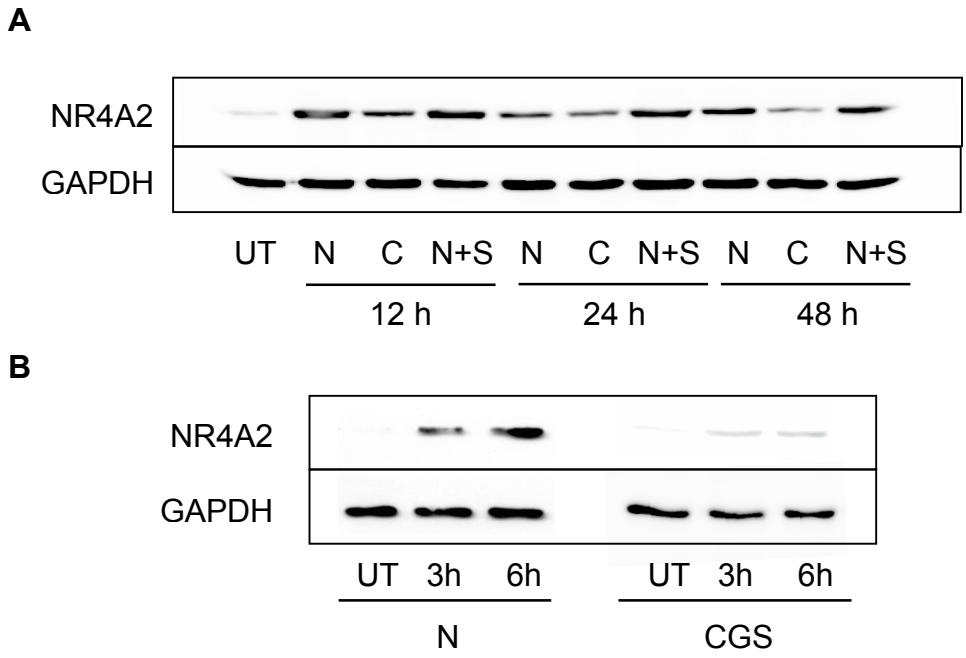

Fig 8. *Western blot analysis of NR4A2 protein. HMC-1 cells were treated with 10μM NECA (N), 10μM CGS-21680 (C) or the combination of 10μM NECA and 10μM SCH-58261 (N+S) for the indicated time points. UT= untreated. GAPDH is included as a loading control.*

Transcriptional activity of NR4A receptors

NR4A orphan receptors bind to well-defined DNA sequences located in promoter regions of various genes and activate their transcription. The nerve-growth factor responsive element (NBRE) is a well-characterized NR4A consensus sequence. Here, we employed an NBRE-luciferase reporter plasmid (Fig 9A) to assess the transcriptional activity of AR-induced NR4A receptors. Figure 9B shows the time-dependent induction of luciferase activity upon treatment with NECA, CGS-21680 or NECA together with SCH-58261. NECA and NECA plus SCH-58261 induced a robust response, which peaked at 24h with 40-fold reporter induction compared to untreated controls and returned to background values at 72h. CGS-21680, in contrast, did not induce luciferase expression significantly.

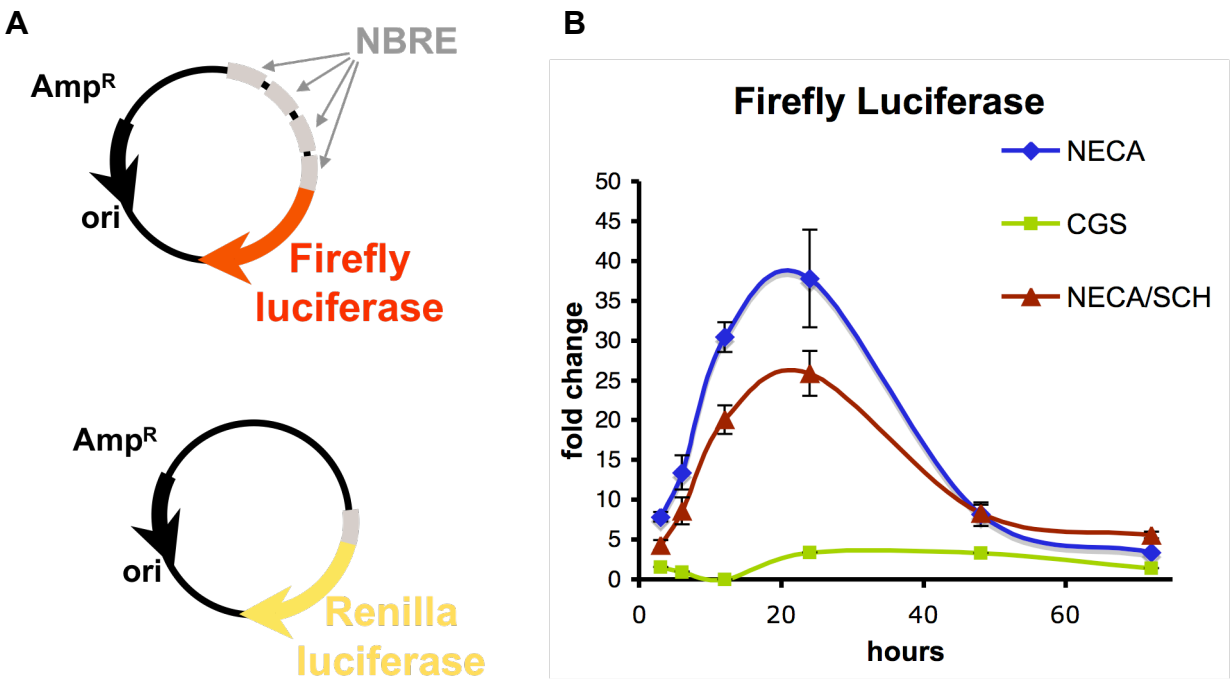

Figure 9. *A) Vector maps of the reporter plasmids. In 4xNBRE-LUC the expression of the firefly luciferase gene is under the control of 4 NBRE repeats. The plasmid Lt-K encodes the renilla luciferase gene, which is consitutively expressed, and was co-transfected with the 4xNBRE-LUC as a transfection control. B) Induction of NR4A-mediated transcriptional activity by AR activation. HMC-1 cells were transfected with the indicated plasmids and treated with 10μM NECA, 10μM CGS-21680 and 10μM NECA together with 10μM SCH-58261. Whole cell lysates were obtained at the indicated times and assessed for luciferase activity.*

Interplay between ARs and PMA/Ionomycin in NR4A2 and NR4A3 signaling

NR4A receptors are immediate responsive genes that can be induced by a variety of stimuli. Recent evidence indicates that NR4A2 induction represents a point of convergence of different cytokine signaling pathways, indicating a common role for this transcription factor in mediating multiple inflammatory signals (Mcevoy et al., 2002). Therefore, we wanted to assess whether the stimulation of ARs could alter the effect of other inflammatory stimuli on NR4A

upregulation and activation. Phorbol 12-myristate 13-acetate (PMA) is a diester of phorbol that is usually employed together with the calcium ionophore Ionomycin (I) for the study of cytokine induction during the inflammatory response in mast cells (and other cell types). Stimulation of HMC-1 cells with the combination of PMA/I for 2h resulted in a very strong induction of NR4A2 and NR4A3 and a concomitant 104-fold increase in their transcriptional activity compared to untreated controls with the NBRE-LUC reporter. In the presence of 10μM NECA, the induction of these orphan receptors was markedly accentuated (more than 1000-fold over baseline controls), with activity levels more than 3-times higher than with PMA/I alone (Fig. 10B). In contrast, the addition of 10μM CGS-21680 to the PMA/I mixture significantly reduced NR4A2 and NR4A3 induction by more than 50% and the NBRE transcriptional activity by around 30%.

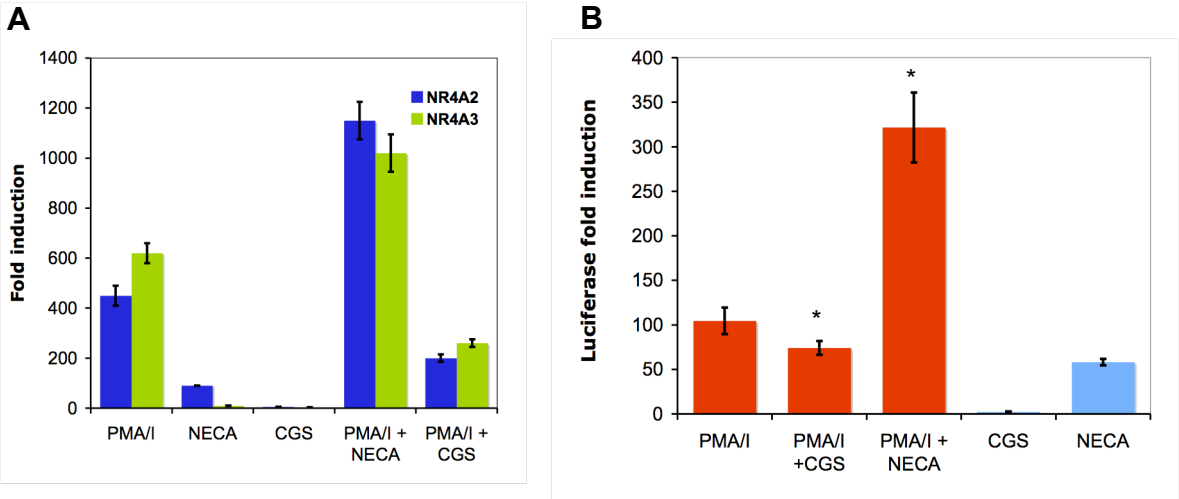

Figure 10. *A) Induction of NR4A2 and NR4A3 transcripts by PMA/I alone or in combination with NECA (PMA/I + NECA) or CGS-21680 (PMA/I + CGS). B) Induction of NR4A-mediated transcriptional activity by PMA/I and AR agonists. Values represent the mean of three measurements ± SEM. Asterisks indicate significant differences ($P < 0.05$) when compared to PMA/I treatment.*

Discussion

There is overwhelming evidence indicating that mast cell mediators, such as cytokines and histamine have a key role in airway inflammation and hyperresponsiveness, and that chronic mast cell activation contributes to the pathophysiology of diverse diseases. In addition, mast cells have been reported to mediate adenosine effects in airways (Hua et al., 2008).

In this study we employed a pharmacogenomics approach for the identification of adenosine receptor subtype-specific cellular signals in human mast cells. As a result of this screening, we have detected a link between AR activation and MIP-1β chemokine secretion in human mast cells. In addition, we have identified the role of AR subtypes in regulating the induction and function of NR4A2 and NR4A3 in the context of inflammatory signaling.

MIP-1-β induction/secretion by NECA in HMC-1 cells

MIP-1β together with other MIP-1 family members plays a central role in acute and chronic inflammation by virtue of their potent modulator of cytokine production and lymphocyte chemoattractant actions. Our genome wide mRNA screening revealed a 4.4-fold upregulation of MIP-1β over basal levels 3h after NECA treatment that was confirmed by real time PCR. Furthermore, treatment with this synthetic adenosine analogue induced a robust MIP-1β release by HMC-1 cells, reaching 834pg/ml of cell culture medium - over 60 times more than basal levels (13pg/ml). To the best of our knowledge, this is the first study establishing a link between AR activation and MIP-1β release in mast cells. Research on this cell type has been mainly focused in degranulation-related events in allergy and asthma. Nevertheless, it is becoming more evident that mast cells contribute to the development of many pathological conditions, including autoimmune disease (Benoist and Mathis, 2002), inflammatory arthritis (Lee et al., 2002) and tumorigenesis (Coussens and Werb, 2001) and they are now being acknowledged as versatile effector cells in innate and acquired immunity – beyond their well-characterized IgE mediated effects.

Our findings suggest that adenosine can contribute to the perpetuation of inflammation by directing the recruitment of inflammatory cells by mast cells. The ELISA analysis also revealed that activation of A_{2A}AR with CGS-21680 induced MIP-1β secretion, up to 515 pg/ml (61% of that by NECA). This finding contests other studies showing that A_{2A}AR activation can limit MIP-1β secretion during inflammation. For example, in LPS-stimulated human neutrophils, the engagement of the A_{2A}AR selectively prevented the expression and release of MIP-1β, TNF-α, MIP-1α, MIP-2α, and MIP-3α (Mccoll et al., 2006). Similarly, activation of A_{2A}AR suppresses macrophage function and reduces the degree of kidney injury during glomerulonephritis via suppression of the glomerular expression of MIP-1β among other chemokines (Garcia et al., 2008). One possible explanation for this discrepancy could be that the relatively high concentration of CGS-21680 employed in this study (10μM) binds not only to the A_{2A}AR (K_i= 27nM) but also the A_3AR (K_i= 67nM), which is highly expressed in HMC-1 cells and has been associated with proinflammatory events. Therefore, it is possible that the MIP-1β induction observed upon CGS-21680 treatment reflects a combined effect on A_{2A} and A_3ARs. Complementary experiments with lower doses of CGS-21680 (which exclusively activates the A_{2A}AR) and A_3AR selective agonists would be required to confirm this hypothesis.

In the context of HIV infection, MIP-1β has been identified as a soluble suppressive factor (HIV-SF) released by immortalized and primary CD8+ T cells (Cocchi et al., 1995). CCR5 is the natural receptor for MIP-1β, MIP-1α and RANTES, and participates in the interaction between HIV-1 and CD 8+ T-cells, an early step in viral uptake and replication. As a consequence of this shared receptor usage, the entrance of HIV-1 (in CCR5-dependent strains) can be blocked by the CCR5 ligands, including MIP-1β. Hydroxyurea and rapamycin are examples of drugs that increase the concentrations of MIP-1β, MIP-1α and RANTES, and it is considered as a suitable approach to inhibit HIV-1 (Heredia et al., 2003). Based on our findings, activation of ARs (alone or in combination with other drugs) may represent a novel strategy to increase MIP-1β release by mast cells, that could potentially affect HIV-1 uptake by CD 8+ T-cells.

Induction of functional nuclear orphan receptors by selective AR activation

NR4A orphan receptors are transcription factors belonging to the superfamily of steroid nuclear hormone receptors that have been associated with different cellular processes such as apoptosis in lymphocytes (Winoto and Littman, 2002), development of dopaminergic neurons (Zetterstrom et al., 1997) and glucose metabolism (Pei et al., 2006). It has been reported that this family of orphan receptors represent NF-κB and LPS-inducible genes in macrophages (Pei et al., 2005) and that NR4A1 expression leads to induction of inflammatory genes, potentiating NF-κB pro-inflammatory signaling by IKKi kinase upregulation (Pei et al., 2006). In addition, NR4A2 has been recognized as an important inducible factor in inflamed synovium and as a target of adenosine-mediated anti-inflammatory effects of methotrexate (Ralph et al., 2005).

The results presented here establish, for the first time, a direct link between AR engagement and activation of two members of the NR4A subfamily of nuclear receptors, namely NR4A2 and NR4A3. Furthermore, we uncover divergent effects between the AR subtypes on the induction of these orphan receptors. First, we demonstrate that AR activation leads to a sharp increase in NR4A2 and NR4A3 mRNA and protein levels (but not of NR4A1) and that this upregulation is multiplied by blocking the A_{2A}AR. Second, we present evidence indicating that the induction of these orphan receptors is accompanied by an increase in their transcriptional activity. Third, we establish that activation of A_{2A}AR not only counterbalances general adenosine effects, but the engagement of this AR can also modulate the induction of NR4A2 by other inflammatory stimuli.

This remarkably high induction of NR4A2 and NR4A3 peaks between 1 and 2 hours after AR stimulation and is accompanied by an increase in their transcriptional activity that returns to background level after 48h, suggesting important transcriptional downstream effects. In contrast to other members of the nuclear hormone receptor superfamily, the crystal structure and NMR data indicate that the ligand-binding pocket of NR4A receptors is covered by

hydrophobic residues. These receptors have been demonstrated to function as ligand-independent constitutively active transcription factors whose activity is controlled at the level of protein expression and post-translational modifications (Codina et al., 2004; Fahrner et al., 1990). For this reason, adenosine's effects on the abundance of these transcription factors could have broad biological implications. On one hand, the activation of pro-inflammatory ARs (i.e. A_{2B} and A_3ARs) in activated inflammatory cells could act as an amplification signal resulting in very high levels of NR4A transcriptional activity and expression of a larger set of NBRE-responsive genes. On the other hand, A_{2A}AR activation could directly downregulate NR4A2 and NR4A3-dependent inflammatory genes.

NR4As also influence the function of other inflammation-associated transcription factors. For example, NR4A1 and 2 form heterodimers with retinoic acid receptor and may influence retinoid signaling (Wallen-Mackenzie et al., 2003). AR activation can therefore affect the number of NR4A-containing complexes. A_{2B} and A_3AR-mediated accumulation of NR4A2 and NR4A3 would translate in a higher proportion of transcriptional complexes containing these orphan receptors. Conversely, A_{2A}AR activation would limit the availability of NR4A2 and NR4A3 for heterodimerization with other TFs. As a result of this interplay, adenosine could affect the nature of these transcriptional complexes and, by extention, the range of transcribed genes.

NR4A receptors can also crosstalk with other TFs and influence their activity, without necessarily interacting with them. A recent study has established that NR4A receptors and the estrogen-related receptors NR3B mutually repress each others transcriptional activity (Lammi et al., 2007). Similarly, NR4A1 has been shown to negatively cross-talk with NF-κB (Harant and Lindley, 2004). Therefore adenosine can indirectly influence gene expression by other TFs by up or downregulating NR4A2 and 3.

In view of the pleiotropic physiological roles of NR4As, adenosine's effect on this group of transcription factors could have broad implications. A screen of all human gene promoters from a curated database of transcriptional start sites revealed 483 candidate genes containing one or two NBREs in their promoter regions (Zhao et al., 2008). Recently, NR4A orphan receptors have been proposed as downstream effectors of inflammatory signaling pathways

in macrophages (Pei et al., 2006). To the best of our knowledge, the role of this subfamily of orphan receptors has not been characterized in mast cells. The results presented here establish a novel signaling axis downstream of ARs involving the NR4A orphan receptor subfamily.

Finally, we provide evidence showing that the nonselective AR activation strongly potentiated PMA/I-mediated induction of NR4As. In contrast, the concomitant treatment of HMC-1 cells with the A_{2A}AR agonist CGS-21680 partially reverted both NR4A2 and NR4A3 upregulation and their transcriptional activation induced by PMA/I, implying that the modulatory effects of A_{2A}AR activation are not limited to adenosinergic proinflammatory signaling. These results suggest a previously undescribed mechanistic association between adenosine and other inflammatory signaling molecules (PMA/I), which may have profound implications for the development of new approaches for the control of inflammatory processes.

Conclusions and outlook

The results presented in this thesis show that AR activation can have broad regulatory effects on human mast cells. First, we show that AR activation induces MIP-1β synthesis and release in HMC-1 cells. Activation of CD30 ligand in mast cells has recently been shown to stimulate degranulation-independent IL-8, MIP-1α and MIP-1β (Fischer et al., 2006), emphasizing the role of mast cells in regulating the inflammatory process. In turn, the secretion of chemokines by mast cells directs the recruitment of inflammatory cells, a pre-requisite for the development of chronic inflammatory diseases. In this context, our findings suggest that adenosine signaling can swiftly induce chemotaxis by MIP-1β release in mast cells.

The second major finding of this study establishes a novel signaling axis downstream of ARs involving the NR4A orphan receptors in human mast cells. The remarkably high induction of NR4A2 and NR4A3 is accompanied by an increase in their activity, suggesting important secondary transcriptional downstream effects. Recent studies have revealed that nuclear receptors interact with other transcription factors and other cell signaling systems (reviewed by Glass and Ogawa, 2006). Therefore, it is likely that adenosine effects on NR4A orphan receptors will affect multiple signaling cascades, eliciting combinatorial secondary transcriptional responses. In this sense, further studies will be necessary to investigate effector molecules downstream of NR4A orphan receptors and possibly of other TFs. The work presented here establishes the use of pharmacogenomics as an efficient approach for the identification of early transcriptional signals upon selective AR activation. Likewise, a genome-wide screening could be of great value for the identification of secondary transcriptional waves at later time points.

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Zusammenfassung

Adenosin ist ein paradoxer Entzündungsmodulator, der einerseits eine entzündungshemmende Wirkung haben kann und andererseits das Entzündungsgeschehen verlängern kann. In diesen Versuchen wurden mit Hilfe der Pharmakogenomik subtypen-spezifische Zellsignale der Adenosinsrezeptoren (AR) in der humanen Mastzelllinie-1 (HMC-1) identifiziert. Es wurden zwei Genprodukte, die bisher nicht mit Adenosin-induzierter Zellvermittlung assoziiert wurden, genauer charakterisiert. Wir stellten fest, dass die AR-Aktivierung in einer Aufregulierung und Sekretion des ‚macrophage inflammatory protein-1 beta' (MIP-1β) resultiert, was auf eine Adenosin-induzierte Modulierung der Chemotaxis über die Mastzellen schliessen lässt. Zudem konnten wir eine Aufregulierung der Transkriptionsfaktoren NR4A2 und NR4A3 beobachten. Dieser Effekt scheint von den AR Subtypen A_{2B} und A_3 vermittelt zu sein, wobei der AR-Subtyp A_{2A} dieser Aktivierung entgegenwirkt. Darüber hinaus erkannten wir, dass eine nicht-selektive AR-Aktivierung die PMA/I-vermittelte Induktion der NR4A potentiert, während dem eine selektive Aktivierung des A_{2A}AR dies teilweise rückgängig macht. Dies deutet darauf hin, dass der modulierende Effekt des A_{2A}AR an andere proinflammatorische Stimuli gebunden sein muss. Insgesamt zeigen diese Ergebnisse, dass die AR-Aktivierung nicht nur über eine direkte Signalwirkung auf entzündungs-assoziierte Faktoren, sondern auch über die chemotaktische Rekrutierung von Entzündungszellen einen regulatorischen Effekt auf humane Mastzellen haben kann.

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